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A behavioral assay to quantify retinal degeneration in *X. laevis*

Jacob Fleming

Candidate for B.S. Degree
in Biology with Honors

April 2008

APPROVED

Thesis Project Advisor: _____
(Dr. Michael Zuber)

Honors Reader: _____
(Dr. Craig Albertson)

Honors Director: _____
Samuel Gorovitz

Date: _____ April 24, 2008

ABSTRACT

Currently, 3.5 million Americans over the age of 40 are blind or visually impaired. Cases of visual impairment can be attributed to a number of causes. Retinitis pigmentosa, glaucoma, and macular degeneration all involve some form of damage to the retina. While medical advances continue to aid those with vision loss, there is currently no cure for visual impairment due to cell death. In the age of stem cells however, there is hope for those with retinal damage.

Seven eye field transcription factors play specific roles in eye development. When injected into developing *Xenopus laevis*, these transcription factors induce the formation of an ectopic eye field and eventually eyes outside of the nervous system. These findings demonstrate that induced retinal stem cells can differentiate and form a functional retina in a developing embryo. However, for these cells to heal the damaged retina, they must also functionally replace dead or dying cells in a mature retina. In order to do this, an assay has to be developed that will allow us to test visual function.

Two behavioral assays developed to assess visual function of *Xenopus laevis* were considered. The OptoMotry system and a second phototropic behavioral assay using color preference as its basis were considered. After various attempts to modify the background color preference assay, a successful design was developed utilizing *Xenopus* characteristic behavioral response.

The bacterial protein nitroreductase converts the relatively nontoxic prodrug CB1954 to a cytotoxic alkylating agent. This protein was used to create a transgenic line of *Xenopus laevis* that drives nitroreductase (NR) expression under the control of the rhodopsin promoter. In the mature retina, expression from the *Xenopus* rhodopsin promoter (XOP) is restricted to the rod photoreceptors. Rod cells, one of two photoreceptor types in the retina (cones are the other), are responsible for vision at low light levels. Additionally, rod cells are generally the first to be degraded when visual loss occurs. When the nitroreductase transgenic line (XOP-NR) is treated with CB 1954, rod death occurs.

The behavioral assay operates under varying levels of light to exploit our ability to selectively ablate rod photoreceptors. If transgenic tadpoles with rod degeneration show a different behavioral response than wild types at low light levels, then this assay can be used to quantify retinal degeneration.

This assay has been successfully used to quantitatively show variance in response of wild types in relation to the quantity of ambient light. The response curve for XOP-NR animals using this assay was delayed for a number of reasons. The current method for producing the transgenic line of *Xenopus* provides approximately 40% XOP-NR animals and 60% wild types. This alone provides the difficult task of sorting out which animals are transgenic. The future of this assay relies on genotyping live animals before testing.

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INTRODUCTION

According to the National Eye Institute, a branch of the National Institutes of Health, nearly 3.5 million Americans over the age of 40 are blind or visually impaired. It is estimated that these numbers will increase 70% by the year 2020 (Congdon et al., 2003). Cases of visual impairment can be attributed to a number of causes. Retinitis pigmentosa, glaucoma, macular degeneration, and diabetic retinopathies all involve some form of damage to the retina. Although retinal damage is not the only cause of visual impairment, it is a large contributor. While medical advances continue to aid those with vision loss, there is currently no cure for visual impairment due to cell death. In the age of stem cells however, there is hope for those with retinal damage.

There are two sources of retinal stem cells: the ciliary marginal zone of the adult eye and the anterior neural plate of a developing embryo. The ciliary marginal zone is a population of retinal stem cells that was long thought to exist only in fish and amphibians (Johns, 1977). Throughout the life of these animals, the retina continues to generate new retinal cells, which are added at the most anterior margin of the retina – the ciliary marginal zone. In 2000, it was found that this area containing retinal stem cells also exists in chickens and mammals (Fischer and Reh, 2000; Moshiri, Close, and Reh 2004). These findings caught the attention of many people hoping to use stem cell therapy to heal the damaged retina. Studies on the ciliary marginal zone may lead to further knowledge about retinal stem cells but it is not the only promising source of these cells.

A number of genes that play a role in early eye development have been identified. It has been shown that seven eye-field transcription factors are expressed in the anterior region of the neural plate during *Xenopus laevis* eye development (Zuber et al. 2003).

The seven eye-field transcription factors, *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *tll*, and *Optx2* interact with one another, forming a complex regulatory network (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Chuang and Raymond, 2001; Goudreau et al., 2002; Lagutin et al., 2001; Lagutin et al., 2003; Loosli et al., 1999; Zuber et al., 1999). When combined in a cocktail and injected into developing embryos, these transcription factors induce the formation of an ectopic eye field and eventually eyes outside of the nervous system (Zuber et al. 2003). This research suggests eye-field transcription factors induce the formation of embryonic retinal stem cells of the eye field. Consistent with this hypothesis, cultured, pluripotent primitive ectoderm expressing eye-field transcription factors is reprogrammed to a retinal stem cell-like fate (Vicizian et al 2008 submitted). When transplanted to embryos from which the endogenous eye-field has been removed, these cells form functional eyes (**Figure 1**).

Figure 1: Schematic of the transplant procedure used to assess retinal stem cell formation. (A) YFP-only, or (B) EFTF RNAs were injected into both blastomeres of two-cell stage embryos from CAG-Venus YFP transgenic *Xenopus laevis*. At stage 9, primitive ectoderm was removed and cultured to the equivalent of stage 15. One half of the eye field was surgically removed from wild-type, stage 15 (host) embryos, and replaced with the donor cells from one half of an explant. The location of the eye field is shaded red.

These findings demonstrate that induced retinal stem cells can differentiate and form a functional retina in a developing embryo. However, for these cells to heal the damaged retina, they must also functionally replace dead or dying cells in a mature retina. In order to do this, an assay has to be developed that will allow us to test visual function.

Two behavioral assays developed to assess visual function of *Xenopus laevis* were considered. The first assay, the OptoMotry system, was previously used to better understand how circadian clocks adapt visual sensitivity (Solessio 2004). Since this method used variable light levels, it appeared suitable for our purposes. A second phototropic behavioral assay using color preference as its basis was also considered (Moriya, Kito, Miyashita, and Asami, 1996).

The developing *Xenopus* tadpole has a characteristic behavioral response when presented with distinct visual stimuli. A useful assay must exploit this response and distinguish between tadpoles with normal and abnormal vision. Both the OptoMotry system and the background color preference assay can, in theory, distinguish *Xenopus* tadpoles with normal and damaged retinas. The first aim of this work was to determine which of these two methods was best suited for this purpose.

The bacterial protein nitroreductase converts the relatively nontoxic prodrug CB1954 to a cytotoxic alkylating agent (Bilsland et al., 2003). The active prodrug induces high frequency DNA cross-linking resulting in proliferation independent cell death (Cui, Gusterson, and Clark 1999).

Gustav A. Engbretson's (Department of Biochemical and Chemical Engineering, Syracuse University) generated a transgenic line of *Xenopus laevis* that drives nitroreductase (NR) expression under the control of the rhodopsin promoter. In the mature retina, expression from the *Xenopus* rhodopsin promoter (XOP) is restricted to the rod photoreceptors. Rod cells, one of two photoreceptor types in the retina (cones are the other), are responsible for vision at low light levels (Conner 1982). Additionally, rod cells are generally the first to be degraded when visual loss occurs. When the nitroreductase transgenic line (XOP-NR) is treated with the prodrug, CB 1954, rod death occurs. In preliminary studies, Drs. Zuber and Patrick Yurco observed rod cell loss in the retina of XOP-NR tadpoles treated with C1954 (**Figure 2**). XOP-NR tadpoles were cultured in 300 μ M CB 1954, for 5 days, fixed and retinal sections stained for rod photoreceptors (XAP2; red), cone photoreceptors (calbindin; green) & nuclei (DAPI; blue). CB 1954 treatment resulted in a dramatic loss of rod cells, with no apparent effect on cone cell survival. Despite these observations, there was concern that CB 1954 treatment may adversely affect other retinal cell types. Previously published work suggests cells adjacent to those expressing nitroreductase might be negatively affected during treatment – termed bystander effect. In place of CB 1954, we have used mitoxantrone (Mtz), which has been

shown to have a diminished bystander effect.

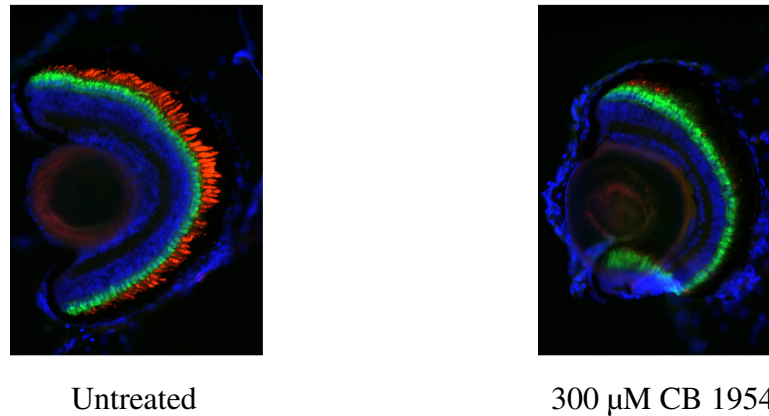


Figure 2: Retinal sections stained for rod photoreceptors (XAP2; red), cone photoreceptors (calbindin; green) & nuclei (DAPI; blue).

Recent results for experiments performed by Rene Choi in the lab indicate Mtz also results in rod specific loss. The ability to selectively ablate rod cells makes it possible to test the behavioral assay to determine if it can distinguish between a normal and degenerated retina. Under low light levels, animals with a healthy retina should have a normal response; Mtz treatment should result in a behavioral change consistent with rod cell loss. The second aim of this work is to determine if the behavioral assay developed in aim one, can be used to distinguish between animals with normal and rod-depleted retinas.

METHODS

a) Animal care

Fertilized eggs were obtained from pigmented *Xenopus laevis* that were primed with 100 Units of pregnant mare serum gonadotropin (VWR, Bridgeport, NJ) 6 days before injecting with 500 U of human chorionic gonadotropin (HCG; Sigma-Aldrich Company, UK) to induce egg laying.

Males were injected in the dorsal lymph sac with 100 Units of HCG 16 -24 hours before harvesting the testes. Eggs were washed two times with 1.0X Marc's Modified Ringer's (MMR; 10X=1 M NaCl; 20 mM KCl; 10 mM MgCl₂ ; 20 mM CaCl₂ ; 50 mM HEPES, pH 7.5). Testes were harvested from a male and put into 1.0X MMR solution. After combining sperm and eggs, embryos were left for two minutes. 0.1X MMR was then added and left to sit for thirty minutes. Upon fertilization, the 1.0X MMR was changed daily with 50 µg/ml gentamicin sulfate (Fisher Scientific, Pittsburgh, PA) in 1.0X MMR. Animals were moved to half-gallon tanks at stage 42-3. The animals were fed 0.5mL Nasco frog brittle/5mL of water every other day. Nasco frog brittle was ground in a motor and pestle before being added to the water. The water in the tanks was changed on a daily basis.

b) OptoMotry system

We attempted to use a vision guided behavior for swimming tadpoles that was previously described (Solessio et al., 2004). Rather than a spinning drum consisting of black and white bars made of paper, the animals were presented with black and white bars projected on four computer screens surrounding the animal as described previously as the OptoMotry system (Umino, Y., Frio, B., Abbasi, M., & Barlow, R. 2006). The animal was placed in the center of the square of screens. Rotating patterns of black and white vertical bars were projected onto the screens and the animal's response was observed through a digital camera (**Figure 3**).

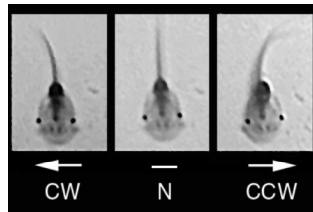


Figure 3: The tadpoles respond correspondingly to different visual patterns. The tail points left in response to a counterclockwise pattern, and the tail points right in response to a clockwise pattern.

c) Background color preference assay

Animals were tested for ten two minute trials with 5 seconds between each trial. After each two-minute trial, a half-gallon flex tank (Nasco, Fort Atkinson, WI) was rotated to flip the black and white sides of the tank. The results were recorded on a digital camera (Sony Handycam DCR-HC42). The test tank consisted of one side being blacked out with electrical tape and the other side covered in tissue paper (**Figure 4**). The inner tank had the wells filled with silgard to avoid animal preference for the indents. To demonstrate that behavior was dependent on vision, the optic nerves were severed using a sterile 26 gauge needle and a number 5 forceps, on anesthetized animals (0.01% ethyl-3-aminobenzoate methanesulfonate salt; Sigma-Aldrich).

QuickTime™ and a
DVD/Video I/O - NTSC decompressor
are needed to see this picture.

Figure 4: The background color preference assay demonstrates the tadpole's preference for the white side of the tank. Healthy wild type tadpoles spend 90 – 95% of their time on the white side.

d) External manipulation of background color preference assay

The design consisted of two pieces of wood that were separated by two 6-inch plastic tubes that were 0.5 inches in diameter (**Figure 5 & 6**). The top layer of wood had a hole cut in it to place a tank in which the animal would rest. A piece of cloth that was half white and half black was run through the piping to cover the outside of the suspended tank. In order to use the design under dark conditions, a light sealed box would cover it to isolate rod vision.

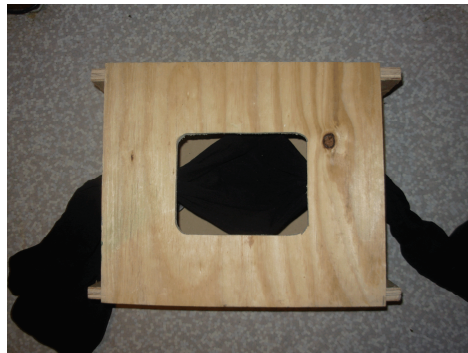


Figure 5: The top view of the design shows the hole for the tank to go in.



Figure 6: The side view of the tank shows the way in which the cloth is pulled across the tank.

e) Automation of background color preference assay

In an attempt to automate the original assay, two computer screens were placed on the long sides of the tank (HP: VP15) and attached to a MacBook Pro running PowerPoint (PP) 10.1.0. The PP document consisted of a slide that was half black and half white. The pattern would flip after two minutes to simulate the pattern change of the original assay. In order to align the two patterns, one of the computer screens was placed upside down.

f) Background color preference assay: dark room

With access to a darkroom, the original color preference assay was designed to operate under minimal light using a fiber optic ring light and a light source. The ring light was attached to a ring stand and placed 0.5 inches from the short side of the tank. The initial light source was powered by 85V of

electricity (Lamda Model No. LLS6120). This light source provided light that was more yellow than white and therefore replaced. The second light source was powered by 115V of electricity and produced white light (Csi/Speco Model No. PSR-7). Various neutral density filters (Omega interference filters) were layered to reduce the amount of light. A digital camera (Sony Handycam DCR-HC42) with an infrared sensor was used to observe the animals under these dark conditions. As above, ten two-minute trials were run for each animal. When an animal failed to show behavior or remained stationary for a single trial that trial was dismissed and the overall average was corrected. Because rods have a maximum response at 520 nm (Solessio et al., 2004), a green light was used with a bandwidth filter with a peak intensity of 520 nm. The amount of light was measured using an optometer with a photometric filter as well as an integral radiometric filter (Graseby Model No. 370).

RESULTS

a) OptoMotry system

Despite spending a number of weeks with this assay, no statistically relevant data was collected. It was difficult to tell which way the animals tail was pointing with the altered method. The animals spent a majority of the time pressed up against the side of the tank, ignoring the projected pattern.

b) Background color preference assay

Six animals spent an average of $92\% \pm 4\%$ of the time on the white side of the test tank (**Figure 7**). When one optic nerve of a stage 51 tadpole was severed, the response was reduced. When both optic nerves were severed, the response was completely random (**Figure 8**).

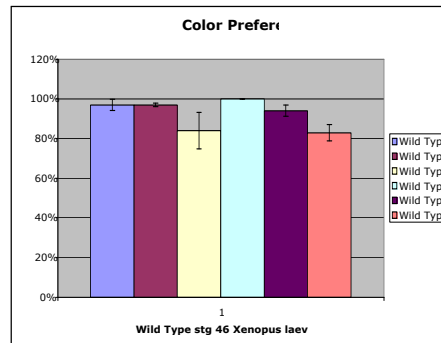


Figure 7: Six wild type animals spent an average of $92\% \pm 4\%$ on the white side of the tank, demonstrating a clear color preference.

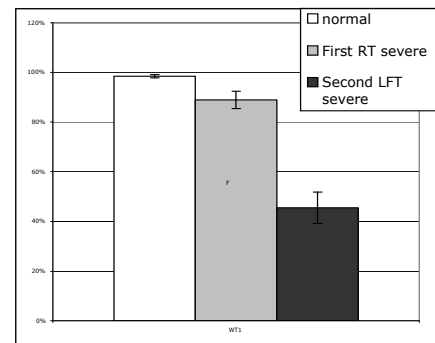


Figure 8: With both eyes, the animal spent $99\% \pm 1\%$ on the white side; with one eye, $89\% \pm 3\%$; with no eyes, $46\% \pm 6\%$.

c) External manipulation of background color preference assay

Due to the fact that the assay has to be run in minimal light to isolate rod vision, I attempted to modify the original assay so it could be manipulated from outside a light sealed box. Despite spending a number of weeks with this design, no statistically relevant data was collected. There were a number of flaws with the design. The cloth was not elastic enough to be pulled back and forth over the tank. The cloth did not remain taut on the outside of the tank. The design was too large to realistically be covered with a light sealed box.

d) Automation of background color preference assay

The first step was to show that the color of the bottom of the tank was not relevant to the behavior of the animal. Six animals spent an average of $87\% \pm 5\%$ on the white side of the tank with a black bottom, $92\% \pm 4\%$ on the white side with a white bottom, and $91\% \pm 3\%$ on the white side of the tank with a gray bottom (**Figure 9**). The short sides of the tank were then shown to be irrelevant for the behavior of the animal. The assay was run with a gray bottom and clear short ends of the tank. The animals spent an average of $92\% \pm 3\%$ of the time on the white side of the tank. With this knowledge, I went

forward with the simulated background change using computer monitors. Initial attempts were unsuccessful; six animals spent an average of $54\% \pm 15\%$ on the white side of the tank. I thought that this might be due to light scatter caused by the monitors. An egg crate was used to concentrate the light on each side of the tank but this remained unsuccessful; six animals spent an average of $58\% \pm 12\%$ on the white side of the tank.

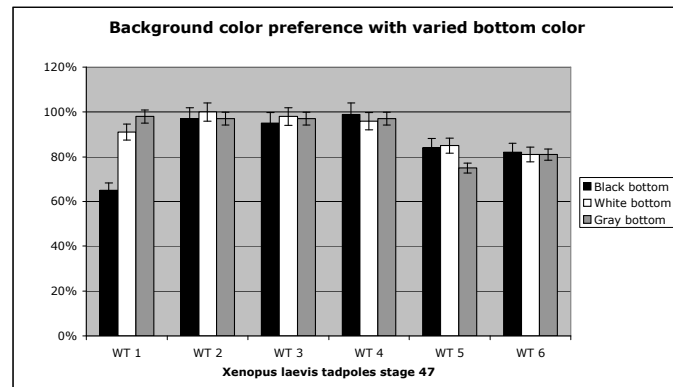


Figure 9: The animals showed no change in behavioral response when the bottom color of the tank was changed. The animals spent an average of $87\% \pm 5\%$ on the white side of the tank with a black bottom, $92\% \pm 4\%$ with a white bottom, and $91\% \pm 3\%$ with a gray bottom.

e) Background color preference assay: dark room

The first light source resulted in six animals spending an average of $78\% \pm 9\%$ on the white side of the tank. Although this result demonstrated response, I changed light sources because the original assay had showed a greater response. The new light source resulted in six animals spending an average of $95\% \pm 2\%$ on the white side of the tank (**Figure 10**). Using a bandwidth filter, the same six animal's response decreased to $66\% \pm 11\%$ (**Figure 11**).

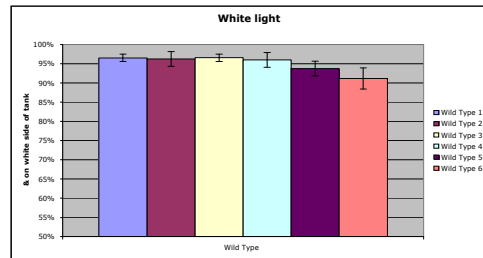


Figure 10: The new light source resulted in a behavioral response that was similar to the original assay. Six animals spent an average of $95\% \pm 2\%$ on the white side of the tank.

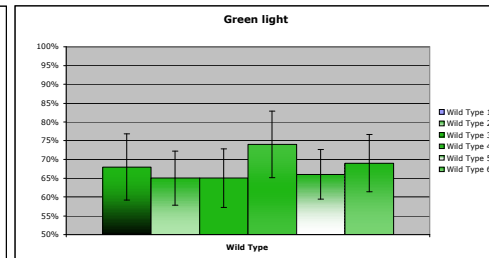


Figure 11: In an attempt to isolate rod vision with green light, a bandwidth filter with a peak intensity of 520 nm was used. Six animals spent an average of $66\% \pm 11\%$ on the white side of the tank.

With the white light results resembling the results of the original assay, I went forward with decreasing the light. Four log units of neutral density filter were used to look for a diminished response. The six animals spent an average of $92\% \pm 3\%$ on the white side of the tank when four log units of neutral density filter were used. To further decrease the light, trials were done with eight, seven, six, and five log units of neutral density filter (**Figure 12**). The amount of light emitted was measured in candela/m² and Watts (**Figure 13**). Additionally, trials were run with no light to test for extraneous light in the dark room. To compensate for variance between animals, an average was taken from the six tadpoles (**Figure 14**).

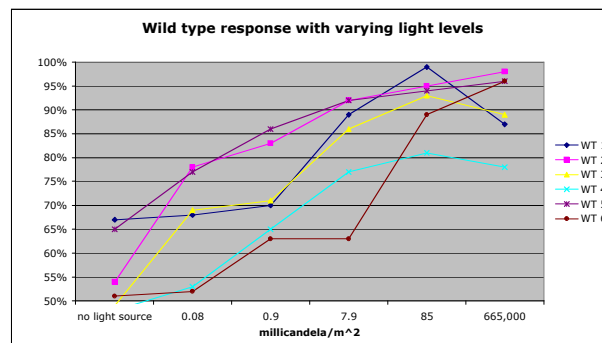


Figure 12: The assay was run under varying conditions of low light. Despite slight variance amongst the animals, the general trend of decreased behavioral response with less light was observed.

Log units of
neutral density
filter

millicandela/m²

nanowatts

0	665,000	14,400
4	85	1.7
5	7.9	0.19
6	0.9	0.017
7	0.08	0.0017
8	0.008	0.00017

Figure 13: The varying levels of light were measured using an optometer. Light levels were recorded in millicandela/m² and nanowatts.

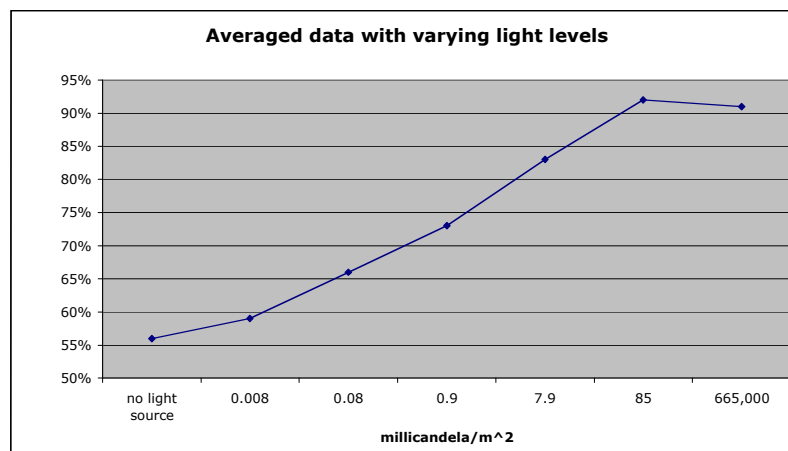


Figure 14: The animal's responses were averaged to compensate for variance between the animals. This made it easier to analyze the responses and to decide which light level would be best used to isolate rod vision while maintaining a consistent behavioral response.

DISCUSSION

The first aim of this work was to develop a behavioral assay that utilized the characteristic behavioral response of *Xenopus laevis* in various levels of light. In order to develop such an assay, past methods were taken into consideration. The OptoMotry system was initially considered for its previous work with *Xenopus* at low light levels (Solessio 2004).

As previously noted, when the tadpoles were stationary in the OptoMotry system, it was difficult to tell which way the tail was pointing. Tadpoles prefer to swim into a current so an attempt to design an artificial

current to place the animal in was drawn up. The idea behind this was while the animal swam into the current, the tail would point more profoundly in one direction in response to the patterns. Stationary animals often spent time up against the edge of the dish, paying little attention to the displayed patterns.

The background color preference assay seemed promising for our purposes because of the simplicity of the design. Attempts at external manipulation and automation of this assay were unsuccessful. The original idea for the external manipulation of the assay failed for a number of reasons. The cloth was not elastic enough to be pulled back and forth over the tank. The cloth did not remain taut on the outside of the tank. The design was too large to realistically cover with a light sealed box. Although the automation of the assay would have made future experiments easier, my design was unsuccessful. The main problem with the computer screens was that there was no definitive division between the white and black sides of the tank. The middle of the tank became gray due to the light scatter of the screens. If there was a way to prevent all light scatter, it is possible that this automated assay would work. I attempted to reduce the light scatter using cardboard dividers but the behavioral response did not improve.

After several failed designs in the dark room, a successful design of the assay was developed using varying light levels. The general trend of a decreased behavioral response with a decreased amount of light was clearly demonstrated with this behavioral assay. Although there was some variance between the different animals, averaging the results provided a distinct response curve. Using a threshold of 75% on the white side of the tank, five

neutral density filters was determined to be the ideal light level to isolate rod vision while maintaining a consistent behavioral response. Five of the six wild type animals performed above the threshold at five neutral density filters, presumably all using rod vision.

In our experiment, we found that several of the animals showed distinct plateaus in their response curves (**Figure 13**). In the curves for wild type two, three, and six, this clear leveling off of response can be seen as the amount of light is decreased. We speculate that this is the lower limit of rod vision before complete vision is lost due to lack of light. To further test this, more animals should be tested to look for similar trends in response. With an increased sample size, this trend can be further analyzed. There are several reasons to account for the variance between the animals.

This is a stage dependent assay as shown in the original background color preference assay (Moriya, Kito, Miyashita, and Asami, 1996). Despite being fertilized on the same day, these animals have varying rates of development, which would explain varying responses. If animals have varying levels of retinal cell populations, response may be affected. The retinas must be sectioned and stained to confirm that there is slight variance between the animals. Additionally, we have seen feeding play a role in the variability of the response. It is impossible to control how much any particular animal chooses to consume but this may account for minor differences in response.

The secondary aim of this work was to determine if the behavioral assay could be used to distinguish between animals with normal and rod-depleted retinas. Although a distinct response curve was produced for wild

types, the response curve for XOP-NR animals using this assay was delayed for a number of reasons. The current method for producing the transgenic line of *Xenopus laevis* provides approximately 40% XOP-NR animals and 60% wild types. This alone provides the difficult task of sorting out which animals are transgenic. When animals were randomly selected, a much greater degree of variance was initially observed. The animals were left to mature for two weeks and then retested. The variance was still much greater than previously seen but several animals were showing the typical response under white light. In order to select animals that were responsive, a pretest was done using white light and zero neutral density filters. The animals that were responsive were retested using five neutral density filters and then treated with Mtz. After five days of treatment, the animals were retested to look for a diminished response.

Although the treatment had not previously killed the animals, we saw a decrease in health in a number of the animals. The one animal that survived did not show a diminished response, suggesting that it was a wild type. If the animal had been transgenic, the depleted rod cells would have resulted in a decrease in response. If genotyping this animal indicates that it is a wild type, then it is likely that the drug is not having a negative impact on the vision of normal animals.

The future of this assay will rely on the ability to sort out the transgenic animals from the wild types before testing them. If XOP-NR animals can be isolated, then the number of trials can be greatly increased. In order to do this, genotyping live animals will be necessary. The major concern with this is that the small tail snip that is required may alter the behavior of the

animal. In order to test for this, tail snips should be taken from a number of wild type animals. These animals can then be tested using the behavioral assay to look for a change in behavior. If the animal's response remain consistent, then this method should be useful for separating the XOP-NR animals.

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WRITTEN SUMMARY OF CAPSTONE PROJECT

According to the National Eye Institute, a branch of the National Institutes of Health, nearly 3.5 million Americans over the age of 40 are blind or visually impaired. The estimate is that these numbers will increase 70% by the year 2020 (Congdon et al., 2003). These cases of visual impairment can be attributed to a number of causes. Retinitis pigmentosa, glaucoma, macular degeneration, and diabetic retinopathies all involve some form of damage to the retina. Although retinal damage is not the only cause of visual impairment, it is a large contributor. While medical advances continue to aid those with visual loss, visual impairment due to cell death currently has no cure. In the age of stem cells, there is hope for those who have damaged retinas.

In order to better understand the concept of healing a damaged retina, some knowledge is required of a healthy retina. The vertebrate retina is composed of a variety of different cells that are organized into layers. The outermost layer of the retina consists of the photoreceptor cells. These are known as rod and cone cells, each with a specific purpose. Rod cells are typically more sensitive and function under less intense light levels. Cone cells are much less abundant in the adult retina and respond to bright light. The innermost layer of the retina is composed of ganglion cells and their nerve fibers. The light received by the eye is sent to the brain via these ganglion cells. The area between the photoreceptor cells and ganglion cells is known as the inner nuclear layer and is composed of a number of varying cell

types (Adler and Farber 1986). For my purposes, the variety of cell types in the inner nuclear layer is not relevant because we have been working specifically on photoreceptor cells. As the photoreceptor cells in the retina deteriorate, vision is lost. It is our hope to use retinal stem cells to replace those cells that no longer function properly.

I have been working in Dr. Michael Zuber's lab at SUNY Upstate Medical University. The Zuber lab works specifically with *Xenopus laevis*, the African clawed frog, and retinal stem cells. When an organism first begins to develop, it is composed of cells that are undifferentiated. That is, each cell can become any part of the body. Retinal stem cells are cells that will eventually become one of the many types of cells that make up the retina. There are two sources of retinal stem cells: the ciliary marginal zone and the anterior neural plate of a developing embryo.

The ciliary marginal zone is a population of retinal stem cells that was thought, for a number of years, to exist only in fish and amphibians (Johns, 1977). As the embryo grows, the retina continues to develop new retinal cells that are added to the front side of the retina, forming the ciliary marginal zone. In 2000, it was found that this area containing retinal stem cells also exists in chickens (Fischer and Reh, 2000). These findings caught the attention of many researchers hoping to use stem cell therapy to heal damaged retinas. Studies on the ciliary marginal zone may lead to further knowledge about retinal stem cells but it is not the only promising source of cells.

There are a number of genes that play a role in eye development. Transcription factors are proteins that bind to our DNA. These proteins help

our DNA and genetic makeup to know what type of cell to eventually become. Seven eye-field transcription factors are expressed during *Xenopus laevis* eye development (Zuber et al. 2003). The seven eye-field transcription factors interact with one another, forming several levels of regulation (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Chuang and Raymond, 2001; Goudreau et al., 2002; Lagutin et al., 2001; Lagutin et al., 2003; Loosli et al., 1999; Zuber et al., 1999). With these eye-field transcription factors, undifferentiated cells can be transformed into retinal stem cells. When these proteins are injected into developing embryos, they cause the formation of ectopic eye fields in *Xenopus* as well as forming ectopic eyes outside of the nervous system (Zuber et al. 2003).

Experiments have demonstrated that induced retinal stem cells can form retinal cell types in developing embryos. The question at hand is, can these same cells replace damaged retinal cells in a mature eye? In order to test the ability of induced retinal stem cells functionally replacing damaged cells, retinal cells must first be degraded in *Xenopus laevis* embryos.

There is a protein that has been shown to be effective in ablating specific cells. When this protein is expressed in a cell, it has the potential to be degraded. The presence of a prodrug is necessary to activate this protein (Bilsland et al., 2003). A prodrug is a drug that is inactive until combined with its activating compound. A line of *Xenopus laevis* embryos was developed that have this protein expression in the rod cells of the retina. When the prodrug is administered, the rods are consequently degraded. With an unhealthy retina, it is theoretically possible to inject retinal stem cells and see

recovery of damaged cells. In order to get to this point, a behavioral test has to be developed that quantitatively shows different behavior between animals with healthy retinas and animals with degraded rod cells. I was given the responsibility to develop such a test.

The ability to isolate the rods for selective cell ablation makes it possible to develop a behavioral test which functions under very low light levels. These low light levels will stimulate rod vision in a healthy retina, while animals with degraded rods will show abnormal behavior due to lack of vision. There have previously been a few different methods to test visual behavior in *Xenopus laevis*. I began with these past methods in hopes of developing something for our purposes.

In 2004, a method was developed to better understand the way in which retinal cells adapt to different times of the day (Solessio 2004). Since this mechanism was used under varying light levels, this was a likely candidate for our purposes. This test projected patterns on a screen for the animals to respond to. The animals would move their tails one way to one pattern and the other way to the alternative pattern. Although this method had previously been successful, we had little success in obtaining statistically relevant results and moved on to another idea.

Looking back at the literature, we found a behavioral test that seemed promising in the *Journal of Behavioral Zoology* (Moriya, Kito, Miyashita, and Asami, 1996). This test showed that *Xenopus laevis* tadpoles prefer to be on the white side of a multicolored tank. The tank we used was half white and half black. When tested, the animals spent 95% of their time on the white side

of the tank. I spent a lot of time in an attempt to automate this test. After several failed attempts, I decided to move forward with running this test in a dark room with varying levels of light.

The light source we used was attached to a fiber optic ring light that was placed over the tank. In order to diminish the amount of light, we used several neutral density filters. A neutral density filter reduces all wavelengths of light evenly and was ideal for our situation. I ran the test under six different light levels with positive results.

When the animals were tested in full light, they spent an average of $91\% \pm 5\%$ of their time on the white side of the tank. I went forward with testing the animals with 4, 5, 6, and 7 neutral density filters. The responses were as follows; $92\% \pm 3\%$, $83\% \pm 6\%$, $73\% \pm 6\%$, $66\% \pm 5\%$, and $56\% \pm 9\%$. This response curve indicates that the animals are slowly losing their behavioral response due to a lack of light. When animals without rods are tested under the same conditions, it is my hypothesis that their response will drop to $50\% \pm 8\%$ while normal animals remain at $83\% \pm 6\%$. Five neutral density filters will be used because it is the lowest light level while still maintaining a consistent response.

The initial hope to produce a response curve for the animals without rods was delayed for a number of reasons. The current method for producing the transgenic line of *Xenopus laevis* provides approximately 40% animals without rods and 60% normal animals. This alone provides the difficult task of sorting out which animals are transgenic. When these animals were randomly selected, a much greater degree of variance was initially observed.

The animals were left to mature for two weeks and then retested. The variance was still much greater than previously seen but several animals were showing the typical response under full white light. In order to select animals that were responsive, a pretest was done using white light and zero neutral density filters. The animals that were responsive were retested using five neutral density filters and then treated with drug. After five days of treatment, the animals were retested to look for a diminished response.

Although the treatment had not previously killed the animals, we saw a decrease in health in a number of the animals. The one animal that survived did not show a diminished response, indicating that it was a normal animal. If genotyping this animal indicates that it is normal, then it is likely that the drug is not having a negative impact on the vision of normal animals.

The future of this test will rely on the ability to sort out the transgenic animals from the wild types before testing them. If animals without rods can be isolated, then the number of trials can be greatly increased. In order to do this, genotyping live animals will be necessary. The major concern with this is that the small tail snip that is required may alter the behavior of the animal. In order to test for this, tail snips should be taken from a number of wild type animals. These animals can then be tested using the behavioral test to look for a change in behavior. If the animal's response remains consistent, then this method should be useful for the animals lacking rods.

Overall, this assay has demonstrated a diminished behavioral response correlated with decreased levels of light. It is our hope that in the future, it can be used to test animals without rods to quantitatively show a greater decrease

in response. These animals can then be injected with retinal stem cells in hopes of regaining full visual function.

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